

Engineering ultra-potent sperm-binding IgG antibodies for the development of an effective non-hormonal female contraception

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Supplementary Information

Materials and Methods

Antibody characterization using SEC-MALS and nano-DSF

SEC-MALS experiments were performed at room temperature using a GE Superdex 200 10/300 column connected to an Agilent FPLC system, a Wyatt DAWN HELEOS II multi-angle light-scattering instrument, and a Wyatt T-rEX refractometer. The flow rate was maintained at 0.5 mL/min. The column was equilibrated with 1X PBS, pH 7.4 containing 200 mg/L of NaN_3 before sample loading. 50-100 μg of each sample was injected onto the column, and data were collected for 50 min. The MALS data were collected and analyzed using Wyatt ASTRA software (Ver. 6).

NanoDSF experiments were performed using a Nanotemper Prometheus NT.48 system. Samples were diluted to 0.5 mg/mL in 1X PBS at pH 7.4 and loaded into Prometheus NT.48 capillaries. Thermal denaturation experiments were performed from 25°C to 95°C at the rate of 1°C/min, measuring the intrinsic tryptophan fluorescence at 330 nm and 350 nm. The melting temperature for each experiment was calculated automatically by Nanotemper PR. Thermcontrol software by plotting the ratiometric measurement of the fluorescent signal against increasing temperature. The aggregation temperature for each experiment was also calculated automatically by Nanotemper PR. Thermcontrol software via the detection of the back-reflection intensity of a light beam that passes the sample.

Stability assay

Briefly, 1.2 mL of Expi293 produced IgG, FIF, FIFF and FFIFF at the 1 mg/mL concentration were prepared using PBS as the solvent. The 1.2 mL of mAbs was split into 6 tubes with 0.2 mL aliquots. Three tubes with 0.2 mL aliquots were stored in 4°C as controls. Another three tubes with 0.2 mL aliquots were stored in 40°C bead bath for 14 days. On each time point: Day 0, Day 7, Day 14, one aliquot was taken out from both 4°C and 40°C for the characterization using SEC-MALS, SDS-PAGE, DSF and whole sperm ELISA.

Whole sperm ELISA

Briefly, half-area polystyrene plates (CLS3690, Corning) were coated with 2×10^5 sperm per well in 50 μL of NaHCO_3 buffer (pH 9.6). After overnight incubation at 4°C, the plates were centrifuged at the speed of 300 g for 20 min. The supernatant was discarded, and the plates were air-dried for 1 hr at 45°C. The plates were washed once with 1X PBS. 100 μL of 5% milk was incubated at RT for 1 hr to prevent non-specific binding of Abs to the microwells. The serial dilution of mAbs in 1% milk was added to the microwells and incubated overnight at 4°C. Motavizumab, a mAb against the respiratory syncytial virus, was constructed and expressed in the laboratory by accessing the published sequence and used as a negative control for this assay¹. After primary incubation, the plates were washed three times using 1X PBS. Then, the secondary Ab, goat anti-human IgG F(ab')₂ Ab HRP-conjugated (1:10,000 dilutions in 1% milk, 209-1304, Rockland Inc.) was added to the wells and incubated for 1 hr at RT. The washing procedure was repeated and 50 μL of the buffer containing substrate (1-Step Ultra TMB ELISA Substrate, Thermo Scientific) was added to develop the colorimetric reaction for 15 min. The reaction was quenched using 50 μL of 2N H_2SO_4 , and the absorbance at 450 nm (signal) and 570 nm (background) was measured using SpectraMax M2 Microplate Reader (Molecular Devices). Each experiment was done with samples in triplicates and repeated two times as a measure of assay variability.

Scanning electron microscopy

Briefly, 20×10^6 washed sperm was centrifuged at 300 g for 10 min. The supernatant was discarded without disturbing the sperm pellet. Next, 200 μL of anti-sperm IgG constructs or

1X PBS was added to the sperm pellet. The Ab-sperm solution was mixed by pipetting and incubated for 5 mins using an end-over-end rotator. 200 μ L of 4% PFA prepared in 0.15 M Sodium Phosphate buffer was added to the Ab-sperm solution and incubated for 10 min using an end-over-end rotator. 50 μ L of fixed sperm samples was filtered through membrane filters (10562, K04CP02500, Osmonics) with 5 mL of 0.15 M Sodium Phosphate buffer. The filter was washed one more time with 0.15 M Sodium Phosphate buffer. Next, the samples were dehydrated in a graded series of alcohol (30% ethanol, 50% ethanol, 70% ethanol, 90% ethanol, 100% ethanol x 3) for 10 minutes each. Filters were transferred to a plate with the transitional solvent, Hexamethyldisilazane (Electron Microscopy Sciences) and allowed to dry after one exchange. Filters were adhered to aluminum stubs with carbon adhesives and samples were sputter-coated with gold-palladium alloy (Au:Pd 60:40 ratio, 91112, Ted Pella Inc.) to a thickness of 3 nm using Cressington Sputter Coater 208 hr. Six random images were acquired for each sample using a Zeiss Supra 25 FESEM with an SE2 Electron detector at 2500X magnification.

Agglutination kinetics assay using low and high concentration of washed semen

Briefly, washed semen at the final concentration of 2×10^6 PM sperm/mL and 50×10^6 PM sperm/mL was prepared. Next, 4.4 μ L of washed semen was added to 4.4 μ L of Ab constructs in 0.2 mL PCR tubes, and mixed by gently pipetting up and down three times over 3 s. Then, 4.4 μ L of the mAb-semen mixture was transferred to chamber slides with a depth of 20 μ m (Cytonix), and a timer was immediately started. At 30s, 60s and 90s time point, CASA measurements were taken focusing on the center of chamber slides. The reduction in percentage of the PM sperm at each time point was computed by normalizing the PM sperm count obtained after mAb-treatment to the PM sperm count obtained after treatment with sperm washing medium control. Experiment involving 1×10^6 PM sperm/mL was evaluated in duplicates on each semen specimen, and the average from the two experiments was used in the analysis. Six independent experiments were performed with n=6 unique semen samples.

Induction of capacitation in washed sperm

Briefly, motile sperm was isolated using the density gradient sperm separation procedure as described above. 2 mL of sperm preparation medium (Origio, Denmark) that was pre-equilibrated under 37°C and 5% CO₂ for 2 hr was used to wash the isolated sperm by centrifuging at 300 g for 10 min. The sperm washing was repeated. The purified sperm pellet was suspended in pre-equilibrated sperm preparation medium and an aliquot was taken for determination of sperm count and motility using CASA. Next, purified sperm at the concentration of 20 million motile sperm/mL was prepared using pre-equilibrated medium. Then, the purified sperm was incubated under 37°C and 5% CO₂ for at least 1 hr to induce capacitation. To confirm capacitation, an aliquot was taken for CASA measurement and CASA's DBT file was further analyzed using CASANOVA software (<https://csbio.unc.edu/CASAnova/index.py>)² to quantify hyperactivated sperm. CASANOVA was used for additional analysis because of the limitation of our Hamilton-Thorne CASA (12.3 version) in quantifying hyperactivated sperm.

Agglutination assay using capacitated sperm

Capacitated sperm were used within an hour of capacitation for the agglutination assay. Briefly, 5 μ L of capacitated sperm was mixed with 5 μ L of mAbs or media control and incubated for 3 mins at RT. Next, 5 μ L of mAb-capacitated sperm was loaded into the chamber slides and CASA measurements were taken to quantify the number of PM sperm. The DBT file from CASA analysis was then uploaded to CASANOVA software (<https://csbio.unc.edu/CASAnova/index.py>) to quantify the number of hyperactivated sperm.

Stability of sperm-mAb agglutinates upon vortexing

Briefly, purified sperm at the concentration of 10 million PM sperm/mL was prepared. 40 μ L of purified sperm was mixed with 40 μ L of mAbs or media control, and incubated for 5 min at RT. CASA measurements were taken to quantify the number of PM sperm after treatment. Next, the mAb- or control- treated sperm was vortexed for 1 min at full speed using _____. CASA measurements were taken again after vortexing. The reduction in percentage of the PM sperm was computed for both non-vortex and vortex conditions by normalizing the PM sperm count obtained after mAb-treatment to the PM sperm count obtained after media-control treatment. Lastly, to confirm the reduction of PM sperm by mAbs under vortex condition was not a mere vortex artifact, the number of PM sperm present in control-treated samples after vortexing was normalized to the PM sperm count present in non-vortex control-treated samples. Five independent experiments were done with at least n=3 unique semen samples. P values were calculated using an unpaired t-test between vortex and non-vortex conditions with Holm-Šidák method. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. Lines indicate arithmetic mean values and standard deviation.

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Stability of sperm-mAb agglutinates over time

Briefly, purified sperm at the concentration of 10 million PM sperm/mL was prepared. 40 μ L of purified sperm was mixed with 40 μ L of mAbs or media control, and incubated for 5 min at RT. CASA measurements were taken to quantify the number of PM sperm after treatment. Next, the mAb- or control- treated sperm were incubated for 24 hr at RT. At three different time points: 2 hr, 4 hr, 24 hr, the sperm-mAb mixtures were gently mixed followed by CASA measurements. The reduction in percentage of the PM sperm was computed for each time point by normalizing the PM sperm count obtained after Ab-treatment to the PM sperm count obtained after media-control treatment. Lastly, to illustrate the decrease in PM sperm count over time in control samples, the number of PM sperm present in control-treated samples of each time point was normalized to the PM sperm count in control-treated samples of initial 5 min incubation. Four independent experiments were done with at least n=3 unique semen samples. P values were calculated using an unpaired t-test between vortex and non-vortex conditions with Holm-Šidák method. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. Lines indicate arithmetic mean values and standard deviation.

Bovine cervical mucus (BCM) capillary tube assay

Briefly, mucus penetration test was performed as previously described³ with some modifications. Estrus bovine cervical mucus (BioIVT, New York) was diluted 2-fold using sperm washing media (Irvine Scientific) prior to loading into the flat rectangular capillary tubes (#2540, VitroCom, New Jersey) of 0.4 mm depth and 5 mm length. The capillary tubes were sealed at one end using critoseal (Ward's Microhematocrit set). The capillary tubes were marked at 1 cm, 3 cm and 4.5 cm from the unsealed end. Next, 150 μ L of whole semen was mixed with 150 μ L of mAbs or media control in 0.5 mL microcentrifuge tubes. Then, the capillary tubes were immediately inserted into the mAb-semen solution. The capillary tube and th were next adjusted to horizontal set up and incubated for 2 hr at RT. The capillary tubes were examined for sperm at the 1 cm, 3 cm and 4.5 cm mark using 20X objective of phase-contrast microscope (Olympus CX41). Manual counting of sperm was performed with sperm categorized as i. Actively motile if forward motility was observed and ii. Shaking/Immotile if sperm appeared trapped in the same location. Total number of sperm present at 1 cm mark in control-treatment conditions was not counted due to the limitation of manual counting against high concentration of sperm.

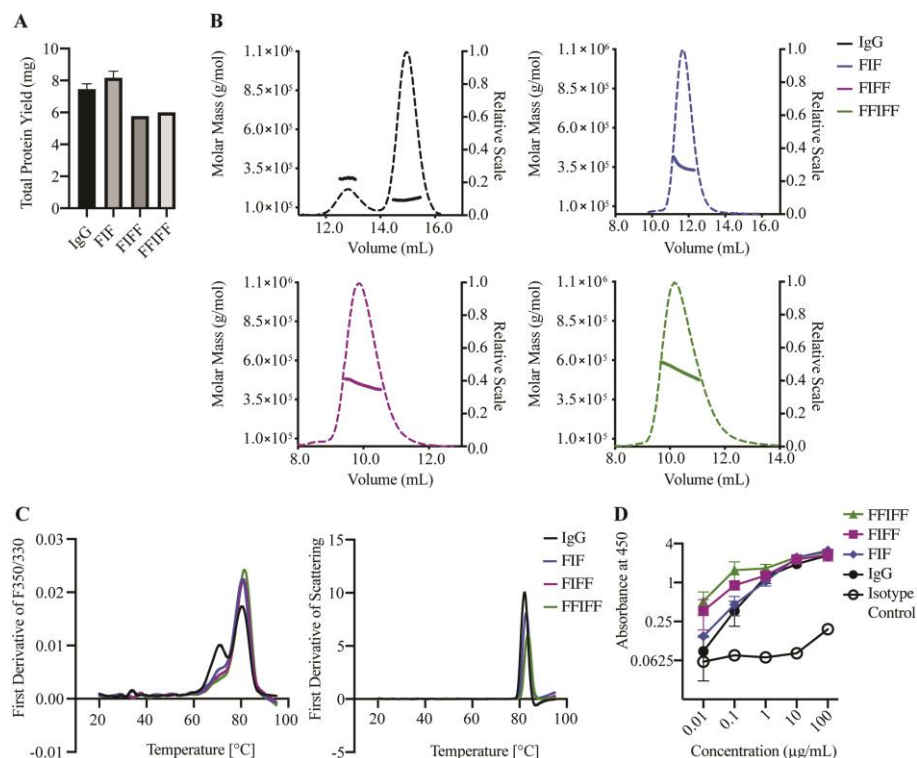


Fig. S1. Additional characterization of highly multivalent anti-sperm IgG antibodies. (A) Production yield of IgG, FIF, FIFF and FFIFF, purified from 90 mL Expi293 transfection. Data were obtained from $n = 2$ independent transfection for IgG and FIF, and one transfection for FIFF and FFIFF. (B) SEC-MALS curves of the parent IgG, FIF, FIFF and FFIFF. Thick lines indicate the calculated molecular mass (left y-axis) and the dotted lines show the homogenous profile (right y-axis) of each antibody. Data collected from one experiment. (C) The melting temperatures (left) and aggregating temperature (right) of the indicated antibodies as determined by nanoDSF by measuring intrinsic fluorescence and changes in back-reflection of proteins respectively. The experiment was performed in duplicates and averaged. (D) Whole sperm ELISA to assess the binding potency of the indicated antibodies to human sperm. Motavizumab (anti-RSV IgG) was used as the isotype control. Data were obtained from $n = 3$ independent experiments with $n = 3$ unique semen donors. Each experiment was performed in triplicates and averaged. Lines indicate arithmetic mean values and standard deviation.

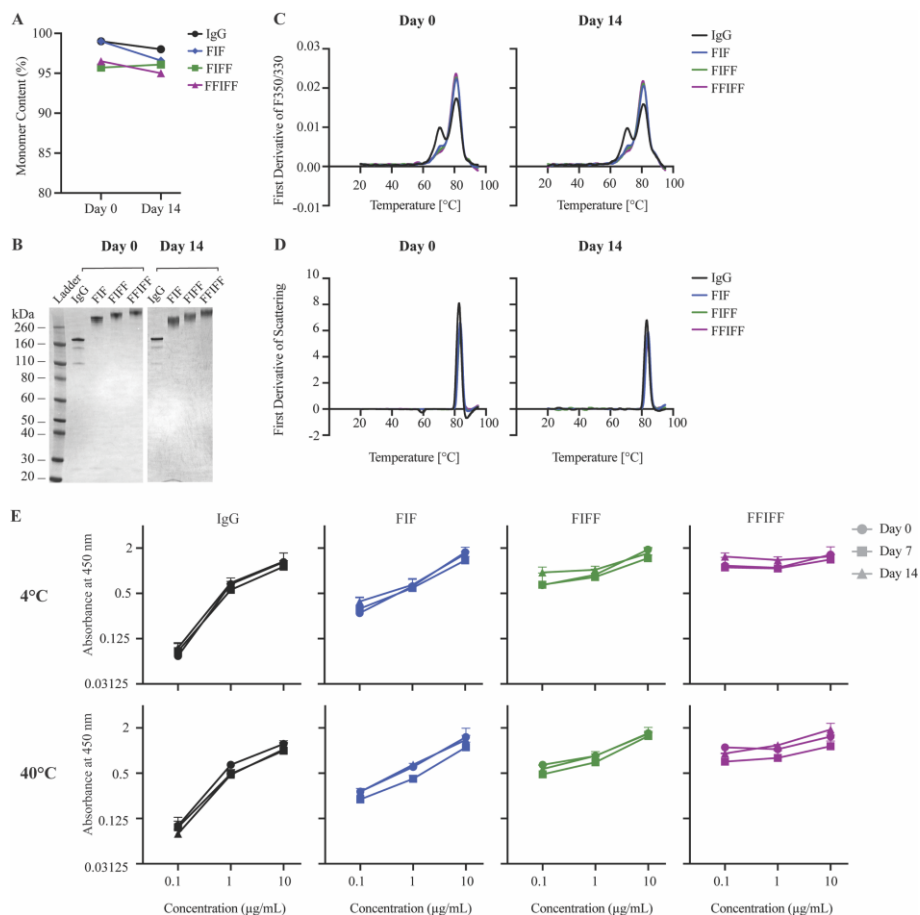


Fig. S2. HM-IgGs exhibit excellent stability at 40°C. (A) Demonstration of the purity and homogeneity of the indicated anti-sperm Abs (100 µg) after storage at 40°C for 14 days using SEC-MALS analysis. (B) Non-reducing SDS-PAGE analysis of the indicated Abs (1 µg) after storage at 40°C for 14 days. (C) The melting temperatures and (D) aggregating temperature (right) of the indicated antibodies after storage at 40°C for 14 days determined by nanoDSF. The experiment was performed in triplicates and averaged. (E) Whole sperm ELISA to assess the sperm-binding potency of the indicated Abs after storage at 40°C for 14 days. ELISA was performed using Abs stored at 4°C for 14 days for reference. Data were obtained from n = 2 independent experiments with n = 2 unique semen donors. Each experiment was performed in triplicates and averaged. Lines indicate arithmetic mean values and standard deviation.

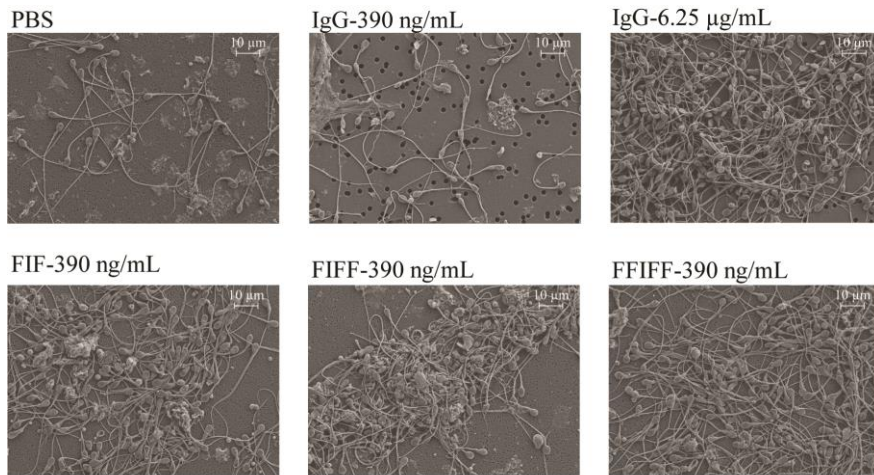


Fig. S3. Scanning electron microscopy images of the agglutinated sperm.
 20 million washed sperm were treated with anti-sperm IgGs for 5 min and fixed using 4% PFA. Images were obtained at 2500X magnification. Scale bar, 10 µm.

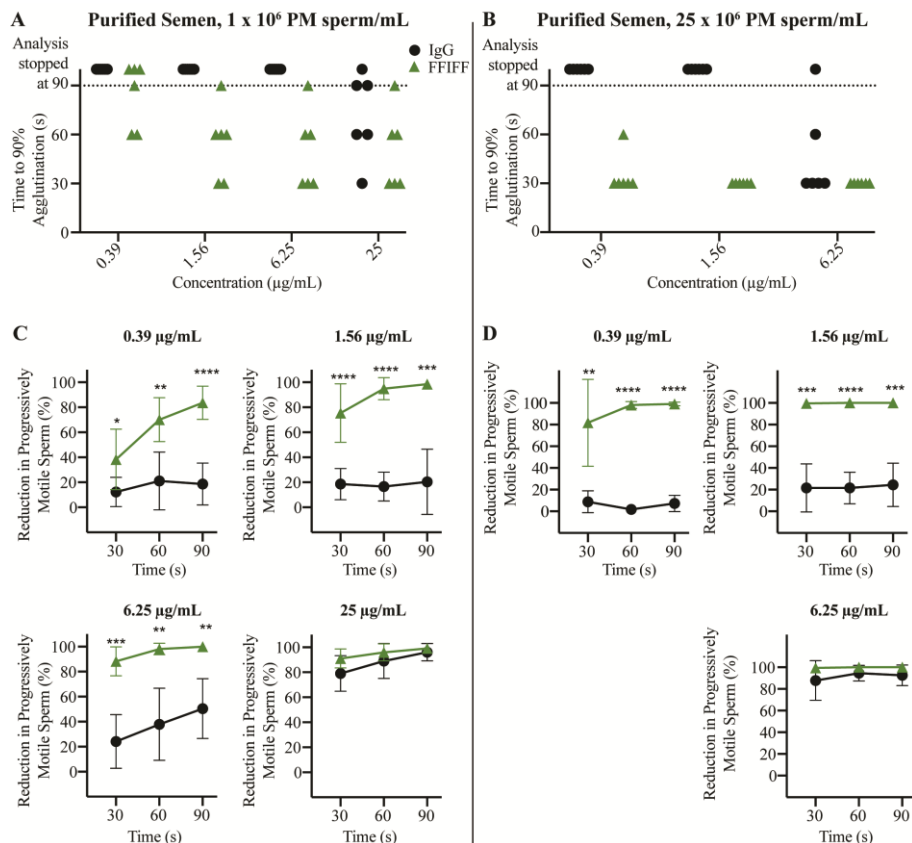


Fig. S4. FFIFF demonstrates faster agglutination kinetics than the parent IgG at both low and high sperm concentration.

(A) Sperm agglutination kinetics of the parent IgG and FFIFF measured by the quantification of time required to achieve 90% agglutination of PM sperm compared to sperm washing media control using 1 x 10⁶ PM sperm/mL and (B) 25 x 10⁶ PM sperm/mL. (C) The rate of sperm agglutination for the parent IgG and FFIFF measured by the reduction in percentage of PM sperm at three different timepoints after Ab-treatment compared to sperm washing media control using 1 x 10⁶ PM sperm/mL and (D) 25 x 10⁶ PM sperm/mL. Data were obtained from n = 6 independent experiments with n = 6 different semen donors. Experiment involving 1 x 10⁶ PM sperm/mL was performed in duplicates and averaged. P values were calculated using a one-tailed t-test. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. Lines indicate arithmetic mean values and standard deviation.

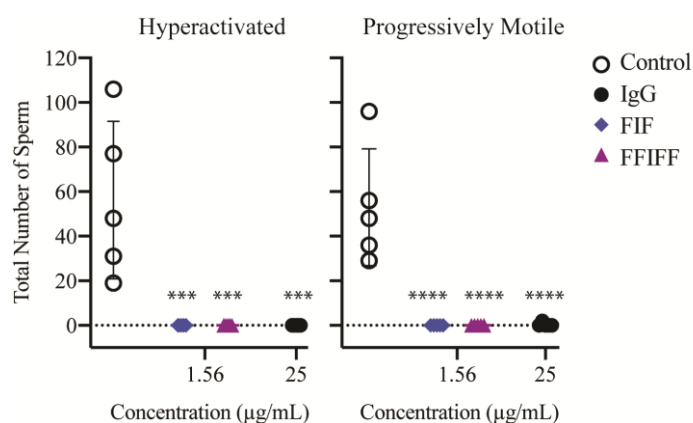


Fig. S5. HM-IgGs exhibit robust agglutination against capacitated sperm.

Sperm agglutination activity of the indicated Abs measured by the CASANOVA-based quantification of the number of sperm that remains Capacitated/Hyperactivated and CASA-based quantification of the number of sperm that remains PM after control- or Ab- treatment. Washed semen at the final concentration of 10×10^6 motile sperm/mL that was capacitated for 1 hr at 37°C and 5% CO₂ was used. Data were obtained from n = 5 independent experiments with n = 3 unique semen donors. P values were calculated using a one-way ANOVA with Dunnett's multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. Lines indicate arithmetic mean values and standard deviation.

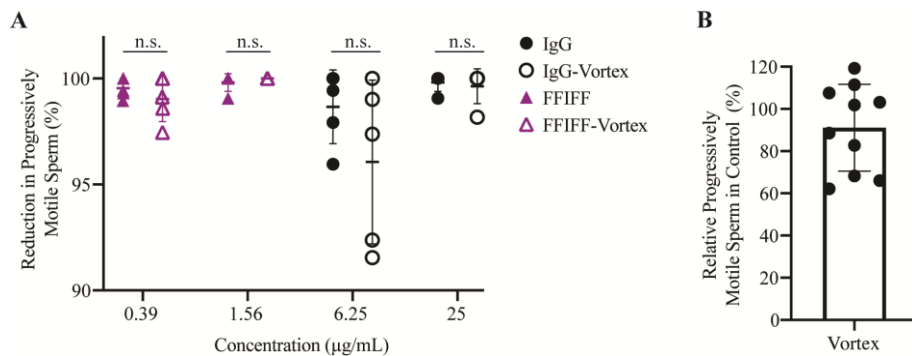


Fig. S6. FFIFF conserves the sperm-agglutination upon mechanical stress.

(A) The stability of mAb-agglutinated sperm upon vortexing measured by the reduction in percentage of PM sperm after Ab-treatment compared to control-treatment. Agglutination was first performed in non-vortex condition as the reference. (B) The number of PM sperm in control-treated sample of post-vortexing condition normalized to the number of PM sperm in control-treated sample of non-vortexing condition. Purified sperm at the final concentration of 5×10^6 PM sperm/mL was used. Data were obtained from $n = 5$ independent experiments with $n = 3$ different semen donors. P values were calculated using paired two-tailed t-tests between vortex and non-vortex conditions. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. Lines indicate arithmetic mean values and standard deviation.

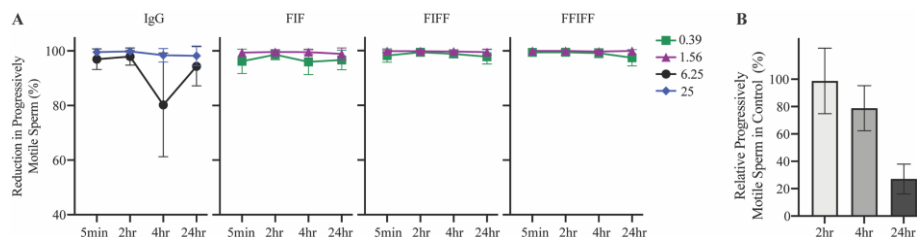


Fig. S7. HM-IgGs conserve the sperm-agglutination for at least 24 hrs.

(A) The stability of mAb-agglutinated sperm over time measured by the reduction in percentage of PM sperm after Ab-treatment compared to control-treatment over three different timepoints. Agglutination activity observed initially at 5 min timepoint is used as the reference. (B) The number of PM sperm in control-treated samples of three different timepoints normalized to the number of PM sperm in control-treated sample of initial 5 min timepoint. Purified sperm at the final concentration of 5×10^6 PM sperm/mL was used. Data were obtained from $n = 4$ independent experiments with $n = 3$ different semen donors. No significant difference was observed for the agglutination activity of all mAbs at different timepoints. For each mAb concentration, P values were calculated using paired one-way ANOVA with Dunnett's multiple comparisons test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. Lines indicate arithmetic mean values and standard deviation.

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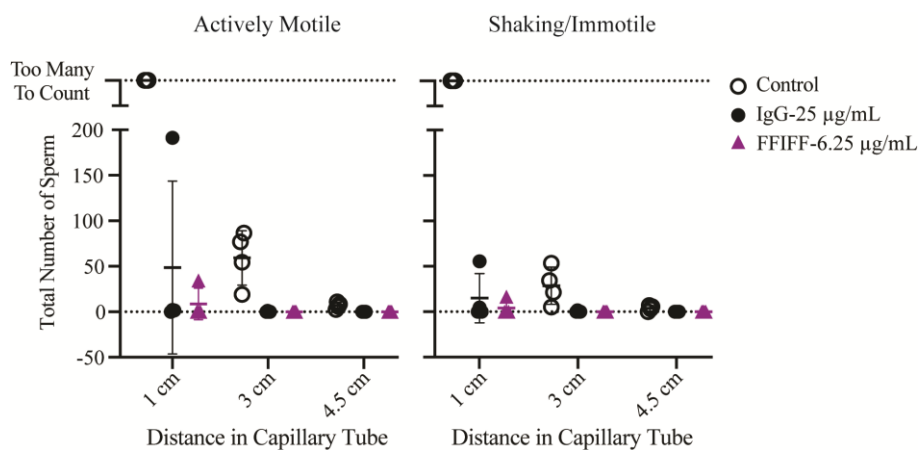


Fig. S8. FFIFF prevent the penetration of vanguard human sperm in bovine cervical mucus. The total number of actively motile and immotile sperm present at three different distances marked on 5 mm BCM-capillary tubes after incubation into semen-mAb mixture for 2 hrs. 25 µg/mL of IgG, 6.25 µg/mL of FFIFF and whole semen was used for the experiment. Data were obtained from n = 4 independent experiments with n = 3 different semen donors. Lines indicate arithmetic mean values and standard deviation.

Table S1. The demographics of the 100 semen samples agglutinated with parent IgM.

Race	Composition (%)
Caucasian	73
African-American	26
Asian	1

Table S2. The demographics of the 20 male donors used in agglutination studies with HM-IgGs.

Race	Composition (%)
Caucasian	60
Hispanic	15
Asian	20
Black	5

Table S3. The sperm motility parameters of the Hamilton-Thorne Ceros 12.3.

Parameter	Value	Parameter	Value
Frames Per Sec	60	Path Velocity (VAP)	25 $\mu\text{m/s}$
No. of Frames	60	Straightness (STR)	80 %
Minimum Cell Size	3 pixels	VAP Cutoff	10 $\mu\text{m/s}$
Default Cell Size	6 pixels	VSL Cutoff	0 $\mu\text{m/s}$
Minimum Contrast	80	Slow Cells	Motile
Default Cell Intensity	20	Standard Objective	10X
Chamber Depth	20 μm	Magnification	1.87

Supplementary videos and captions.

Videos S1-5: Videos of sperm agglutination with ASAs.

Video S1. Sperm remains freely motile upon treatment of whole semen with media control.

Video S2. Sperm are slowly agglutinated upon treatment of whole semen with 12.5 µg/mL of IgG.

Video S3. Sperm are immediately agglutinated upon treatment of whole semen with 1.56 µg/mL of FIF.

Video S4. Sperm are immediately agglutinated upon treatment of whole semen with 1.56 µg/mL of FIFF.

Video S5. Sperm are immediately agglutinated upon treatment of whole semen with 1.56 µg/mL of FFIFF.

Videos S6-10: Videos of muco-trapping of sperm with ASAs.

Video S6. Fluorescently labeled individual motile sperm are not trapped by control IgG 25 µg/mL in CVM.

Video S7. Fluorescently labeled individual motile sperm are trapped by anti-sperm IgG 25 µg/mL in CVM.

Video S8. Fluorescently labeled individual motile sperm are trapped by anti-sperm FIF 25 µg/mL in CVM.

Video S9. Fluorescently labeled individual motile sperm are trapped by anti-sperm FIFF 25 µg/mL in CVM.

Video S10. Fluorescently labeled individual motile sperm are trapped by anti-sperm FFIFF 25 µg/mL in CVM.